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(57) Abstract

A vaccine comprising a liposome preparation including at least one B-cell malignancy-associated antigen, IL-2, alone or in combination with at least one other cytokine, and at least one type of lipid molecule, is useful in a method of inducing humoral and cellular immune responses against malignant B-cells in a mammal.

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VACCINE FOR B-CELL MALIGNANCIES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to methods for inducing humoral and cellular immune responses against malignant B cells. In particular, this invention is directed to methods for producing an integrated immunologic response against tumor cells using antigens that are associated with a B-cell malignancy.

2. Background

One of the major goals of immunotherapy is to harness a patient's immune system against tumor cells or infectious organisms. With regard to cancer therapy, the objective is to direct the patient's immune system against tumor cells by targeting antigens that are associated with tumor cells, but not normal ccunterparts. These tumor associated antigens (TAAs) have been difficult to identify. Certain tumor cells express antigens that are normally not expressed, or expressed at very low levels, in adult life, although they are present during fetal development. One example of such oncofetal TAAs is α-fetoprotein, which is expressed by liver cancer cells. Another oncofetal TAA is carcinoembryonic antigen (CEA), which is expressed in most adenocarcinomas of entodermally-derived digestive system epithelia, as well as in breast tumor cells and non-small-cell lung cancer cells. Thomas *et al., Biochim. Biophys. Acta 1032*: 177 (1990).

The administration of anti-idiotype antibodies (Ab2s) mimicking TAAs represents a promising approach to cancer immunotherapy. Goldenberg, *Amer. J. Med. 94*: 297 (1993). Ab2s are antibodies directed against the variable regions of conventional antibodies (Ab1). Certain Ab2s (termed "Ab2ß", "anti-idiotype" or "internal-image" antibodies) can mimic the three- dimensional structure of the nominal antigen, and thus Ab2 and antigen can bind with the same regions of the Ab1-combining site. Jerne *et al., EMBO J. 1*: 243 (1982); Losman *et al., Int. J. Cancer 46*: 310 (1990); Losman *et al., Proc. Nat'l Acad. Sci. USA 88*: 3421 (1991); Losman *et al., Int. J. Cancer 56*: 580 (1994). Individuals immunized with Ab2ß can develop anti-anti-antibodies (Ab3), some of which can bind the nominal antigen.

The antigen mimicry properties of anti-idiotype antibodies have led to the use of Ab2ß as surrogate antigens (or idiotype vaccines), when the nominal antigen is not readily available or when the host is tolerant to the nominal antigen. In experimental systems, immunization with Ab2ß mimicking certain TAA creates specific immunity to the TAA and protect against subsequent tumor growth. See, for example, Nepom *et al., Proc. Nat'l Acad. Sci. USA 81*: 2864 (1984); Raychaudhuri *et al., J. Immunol. 139*: 271 (1987). Similarly, anti-idiotype vaccines have been developed against infectious organisms, such as *Streptococcus pneumoniae* [McNamara *et al., Science 226*: 1325 (1984)], hepatitus B virus [Kennedy *et al., Science 223*: 930 (1984)], *Escherichia coli* K13 [Stein *et al., J. Exp. Med. 160*: 1001 (1984)], *Schistosomiasis mansoni* [Kresina *et al., J. Clin. Invest. 83*: 912 (1989)], and Moloney murine sarcoma virus [Powell *et al., J. Immunol. 142*: 1318 (1989)].

However, the usefulness of this approach is limited. Cancer patients receiving an anti-TAA of animal origin will usually produce antibodies to the Ab1 and these anti-immunoglobulin antibodies include Ab2. Herlyn *et al., J. Immunol. Methods 85*: 27 (1985); Traub *et al.,*

Cancer Res. *48*: 4002 (1988). The anti-idiotype response also may include the generation of T cells (T2). Fagerberg *et al.*, *Cancer Immunol. Immunother.* *37*: 264 (1993). Moreover, Ab2 may subsequently induce a humoral and cellular anti-anti-idiotypic response, Ab3 and T3, respectively, which may recognize the same epitope as Ab1. *Id.* This is a problem because it can reduce the effectiveness of the immune response.

Thus, an opportunity exists to provide an approach to immunotherapy utilizing both humoral and cellular immune systems. The present methods to provoke an integrated response against tumor cells, particularly malignant B cells, is an initial result of this approach.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a vaccine and method of treatment by inducing humoral and cellular immune responses against malignant B cells, in particular lymphoma, chronic lymphocytic leukemia and multiple myeloma. The vaccine comprises a liposomal preparation that incorporates at least one B cell malignancy associated antigen, at least one cytokine, and at least one type of lipid molecule. This combination therefore provides a novel and more potent vaccine formulation for B cell malignancies. The B-cell malignancy-associated antigen is preferably derived from the patient to be treated and thus the vaccine will be directed against the patient's malignant B-cells.

Thus, in one embodiment, the invention provides a vaccine comprising a liposome preparation comprising (1) at least one B-cell malignancy-associated antigen; (2) IL-2, alone or in combination with at least one other cytokine; and (3) at least one type of lipid molecule.

In another embodiment, the B-cell malignancy-associated antigen comprises all or part of an antibody associated with or produced by a malignant B-cell. Such malignant B-cells include those associated with lymphoma,

chronic lymphocytic leukemia and multiple myeloma. In a further embodiment, the vaccine of the invention additionally comprises a tumor-associated antigen that is not an antibody or antibody fragment. Examples of such additional TAAs include, e.g., MUC-1, Epstein Barr Virus (EBV) antigen or an antigen associated with Burkitt's lymphoma.

In an alternative embodiment, the vaccines of the invention additionally comprise normal B-cell antigens such as HLA antigens.

In another embodiment, the vaccine of the invention additionally comprises a another cytokine; examples of additional cytokines include M-CSF, GM-CSF and IFN-gamma.

The vaccines of the invention comprise at least one lipid molecule selected from the group consisting of phospholipid, cholesterol, and glycolipid and derivatives of these lipids. In a further embodiment, the vaccines of the invention also comprise a carrier protein, e.g., albumin.

In another embodiment, a method for inducing humoral and cellular immune responses against malignant B-cells in a mammal is provided, comprising administering to said mammal a vaccine comprising a liposome preparation comprising (1) at least one B-cell malignancy-associated antigen; (2) IL-2, alone or in combination with at least one other cytokine; and (3) at least one type of lipid molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B and 1C show features of liposomes prepared according to the invention.

Figure 2 shows survival rates of immunized and control mice according to Example 2.

Figure 3 shows survival rates of immunized and control mice according to Example 3.

Figure 4 shows survival rates of immunized and control mice according to Example 5.

Figure 5 shows anti-idiotype titers as a function of IL-2, according to Example 6.

Figure 6 shows T-cell proliferation as a function of vaccine dose, according to Example 7.

Figure 7 shows survival rates for immunized and control mice according to Example 8.

DETAILED DESCRIPTION

The vaccine is composed of three categories of molecules:

1. At least one B-cell malignancy associated antigen. Such an antigen is preferably an antibody, or a fragment of an antibody.
2. Cytokine, in the form of IL-2 alone or IL-2 plus one or more different cytokines such as IL-2, M-CSF, GM-CSF or IFN-gamma.
3. At least one type of lipid molecule, in the form of one or more phospholipids alone or in combination with one or more different lipids such as cholesterol.

The vaccine structure comprises a microscopic vesicle composed of lipid(s), cytokine(s) and at least one B-cell malignancy-associated antigen. The vaccine of the invention may also include an adjuvant or carrier protein, such as albumin.

1. Definitions

An **antigen** is a substance that, upon introduction into a vertebrate animal, stimulates the production of antibodies.

An **idiotype** is an antigenic determinant of the variable region of an antibody.

A **B-cell malignancy associated antigen** is a molecule produced by or associated with malignant B cells, but which is not normally expressed, or is expressed at very low levels, by a non-malignant B-cell. Examples of B-cell malignancy associated antigens include antibodies,

antibody fragments produced by malignant B-cells, and other non-antibody antigens produced by or associated with malignant B-cells. Antibody fragments according to the invention normally comprise an idiotype.

A **tumor cell associated antigen (TAA)** is a molecule produced by or associated with malignant cells, but is not normally expressed, or expressed at very low levels, by a non-malignant cell.

A **lipid** is any of a group of biochemicals which are variably soluble in organic solvents, such as alcohol. Examples of lipids include phospholipids, fats, waxes, and sterols, such as cholesterol.

A **vaccine** is a material that is administered to a vertebrate host to immunize the host against the same material. Typically, a vaccine comprises material associated with a disease state, such as viral infection, bacterial infection, and various malignancies.

2. Production of Antigen

a. B-cell malignancy-associated antibodies and antibody fragments

An antigen according to the present invention can be an antibody molecule produced by the malignant B-cell or a fragment of such an antibody. In lymphoma, the antibodies associated with B cells typically contain a transmembrane domain. In chronic lymphocytic leukemia, such antibodies also have a transmembrane domain. In multiple myeloma, the malignant B-cells often secrete fragments of antibodies.

In one embodiment, these antibodies will be derived from the patient to be treated for B-cell malignancy. The antibodies can be extracted from a sample of tissue containing malignant B-cells which has been obtained from a patient with a B-cell malignancy. Typically such a tissue sample will be taken from the lymph nodes of the patient. In patients with multiple myeloma, antibodies can be extracted from the patient's serum and urine. It is known in the art that certain antibody light chain

molecules are associated with multiple myeloma. One example of such a protein is a Bence-Jones protein. Using protein extraction and purification procedures well known to those of skill in the art, the B-cell antibodies can be isolated and purified. Such isolation and purification techniques include affinity chromatography, for example with protein-A sepharose, size exclusion chromatography and ion-exchange chromatography. See, for example, CURRENT PROTOCOLS IN IMMUNOLOGY, VOL 1, pages 2.7.1-2.7.12 (John Wiley & Sone 1991), METHODS IN MOLECULAR BIOLOGY, VOL. 10, pages 79-104 (The Humana Press, Inc. 1992). It is also known in the art that three major idiotypes are associated with chronic lymphocytic leukemia.

In another embodiment, the patient tissue sample containing malignant B cells will be used to create monoclonal antibodies <u>in vitro</u>. Typically, malignant tissue, containing malignant B-cells is fused with a mouse cell line to produce a hybridoma cell line that will produce a malignant B-cell-associated antibody. Techniques for making monoclonal antibodies are well known to those of skill in the art. See, for example, Kohler and Millstein, *Nature* 256: 495 (1975) and CURRENT PROTOCOLS IN IMMUNOLOGY, VOL 1, pages 2.5.1-2.6.7 (John Wiley & Sone 1991)

In one embodiment, the antigen will comprise a fragment of an antibody produced by malignant B-cells. Normally, such a fragment will comprise an idiotype associated with the malignant B-cell. An antibody fragment according to the invention includes (A) a "half antibody" molecule, *i.e.*, a single heavy:light chain pair, and (B) an enzymatically cleaved antibody fragment, such as the univalent fragments Fab and Fab', the divalent fragment $F(ab')_2$, and a single or double chain Fv fragment. An Fv fragment of an antibody is made up of the variable region of the heavy chain (Vh) of an antibody and the variable region of the light chain of an antibody (Vl).

In accordance with the present invention, fragments within the invention can be obtained from an antibody by methods that include digestion with proteases such as pepsin or papain and/or cleavage of disulfide bonds by chemical reduction. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent Nos. 4,036,945 and 4,331,647 and references contained therein, which patents are incorporated herein in their entireties by reference. Also, see Nisonoff *et al.*, *Arch Biochem. Biophys.* *89*: 230 (1960); Porter, *Biochem. J.* *73*: 119 (1959), Edelman *et al.*, in METHODS IN ENZYMOLOGY VOL. 1, page 422 (Academic Press 1967), and Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Alternatively, antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer such as those supplied commercially by Applied Biosystems, Multiple Peptide Systems and others, or they may be produced manually, using techniques well known in the art. See Geysen *et al.*, *J. Immunol. Methods* 102: 259 (1978). Direct determination of the amino acid sequences of the variable regions of the heavy and light chains of the antibodies according to the invention can be carried out using conventional techniques.

Proteolytic cleavage of an antibody can produce double chain Fv fragments in which the Vh and Vl regions remain non-covalently associated and retain antigen binding capacity. Double chain Fv fragments also can be produced by recombinant expression methods well known in the art. See Skerra *et al.*, *Science* 240: 1038 (1988),

and King *et al.*, *Biochemical J.* 290: 723 (1991). Briefly, the amino acid sequence of the variable regions of the heavy and light chains of antibodies according to the invention can be obtained by direct amino acid sequencing using methods well known to those in the art. From this amino acid sequence, synthetic genes can be designed which code for these variable regions and they can both be inserted into an expression vector. Two polypeptides can be expressed simultaneously from a mammalian or bacterial host, resulting in formation of an active Fv fragment.

An antigen of the present invention also can be a "single chain antibody," a phrase used in this description to denote a linear polypeptide that binds antigen with specificity and that comprises variable or hypervariable regions from the heavy and light chain chains of an antibody. Other single chain antibodies according to the invention can be produced by conventional methodology. The Vh and Vl regions of the Fv fragment can be covalently joined and stabilized by the insertion of a disulfide bond. See Glockshuber, *et al.*, *Biochemistry* 1362 (1990). Alternatively, the Vh and Vl regions can be joined by the insertion of a peptide linker. A gene encoding the Vh, Vl and peptide linker sequences can be constructed and expressed using a recombinant expression vector. See Colcher, *et al.*, *J. Nat'l Cancer Inst.* 82: 1191 (1990). Amino acid sequences comprising hypervariable regions from the Vh and Vl antibody chains can also be constructed using disulfide bonds or peptide linkers, as described herein.

Another form of an antibody fragment is a peptide constituting a single complementarity-determining region (CDR). CDR peptides, such as CDR3, ("minimal recognition units") can be obtained by constructing and expressing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick

et al., Methods: A Companion to Methods in Enzymology 2: 106 (1991).

b. Non-malignancy associated B-cell antigens

The vaccines of the invention can also comprise B-cell antigens which are not specifically associated with malignant B-cells ("non-malignancy associated B-cell antigens"). Examples of these antigens are known in the art and include CD19, CD20, CD21, CD22, CD23, CD25, CD5, and FMC7. Foon, K. *Stem Cells* 13(1):1-21 (1995). Also included in this group are class 1 and class 2 HLA antigens (histocompatibility molecules). Class 1 HLA antigens are also found on almost all other mammalian cells.

c. Other tumor-associated antigens (TAAs)

The vaccines of the invention can additionally comprise other TAAs. Examples of such tumor-associated antigen are MUC-1, EBV antigen and antigens associated with Burkitt's lymphoma.

3. Preparation of Liposome

Liposomes are microscopic vesicles that consist of one or more lipid bilayers surrounding aqueous compartments. See, generally, Bakker-Woudenberg *et al., Eur. J. Clin. Microbiol. Infect. Dis. 12 (Suppl. 1)*: S61 (1993), and Kim, *Drugs 46*: 618 (1993). Liposomes are similar in composition to cellular membranes and as a result, liposomes generally can be administered safely and are biodegradable. Depending on the method of preparation, liposomes may be unilamellar or multilamellar, and liposomes can vary in size with diameters ranging from 0.02 μm to greater than 10 μm. A variety of agents can be encapsulated in liposomes: hydrophobic agents partition in the bilayers and hydrophilic agents partition within the inner aqueous space(s). See, for example, Machy *et al.*, LIPOSOMES IN CELL BIOLOGY AND PHARMACOLOGY (John Libbey 1987), and Ostro *et al., American J. Hosp. Pharm. 46*: 1576 (1989).

Liposomes can adsorb to virtually any type of cell

and then slowly release the encapsulated agent. Alternatively, an absorbed liposome may be endocytosed by cells that are phagocytic. Endocytosis is followed by intralysosomal degradation of liposomal lipids and release of the encapsulated agents. Scherphof *et al.*, *Ann. N.Y. Acad. Sci. 446*: 368 (1985).

Among liposome vectors, cationic liposomes are the most studied, due to their effectiveness in mediating mammalian cell transfection *in vitro*. They are often used for delivery of nucleic acids, but can be used for delivery of other therapeutics, be they drugs or hormones.

Cationic lipids are not found in nature and can be cytotoxic, as these complexes appear incompatible with the physiological environment *in vivo* which is rich in anionic molecules. Liposomes are preferentially phagocytosed into the reticuloendothelial system. However, the reticuloendothelial system can be circumvented by several methods including saturation with large doses of liposome particles, or selective macrophage inactivation by pharmacological means. Claassen *et al.*, *Biochim. Biophys. Acta 802:* 428 (1984). In addition, incorporation of glycolipid- or polyethelene glycol-derivatised phospholipids into liposome membranes has been shown to result in a significantly reduced uptake by the reticuloendothelial system. Allen *et al.*, *Biochim. Biophys. Acta 1068*: 133 (1991); Allen *et al.*, *Biochim. Biohys. Acta 1150*: 9 (1993).

Anionic liposomal vectors have also been examined. These include pH sensitive liposomes which disrupt or fuse with the endosomal membrane following endocytosis and endosome acidification.

Liposome complexes are sometimes targeted to the cell type or tissue of interest by the addition to the liposome preparation of a ligand, usually a polypeptide, for which a corresponding cellular receptor has been identified. An example of a cell receptor that can be targeted is the folate receptor which has recently been

identified as a prominent tumor marker, especially in ovarian carcinomas. KB cells are known to vastly overexpress the folate receptor. Campbell *et al.*, **Cancer Res.** 51: 6125-6132 (1991). Yet other targeting ligands have been examined for liposome targeting including transferrin, protein A, ApoE, P-glycoprotein, α_2-macroglobin, insulin, asiolofetuin, asialoorosomucoid, monoclonal antibodies with a variety of tissue specificity, biotin, galactose or lactose containing haptens (monovalent and tri-antennary), mannose, dinitrophenol, and vitamin B12. The ligands are covalently conjugated to a lipid anchor in either pre-formed liposomes or are incorporated during liposome preparation. Lee and Low **J. Biol. Chem.** 269: 3198-3204 (1994) and Lee and Low **Biochim. Biophys. Acta** 1233: 134-144 (1995).

Synthetic peptides are sometimes incorporated into DNA/liposome complexes to enhance their activity, or to target them to the nucleus. For example, in order to gain access to the cytoplasm, the molecule to be delivered must overcome the plasma membrane barrier. In nature, viral fusion peptides facilitate the delivery into the cytoplasm by promoting viral membrane fusion with the plasma membrane. For recent reviews on this subject see Stegmann *et al.*, *Ann. Rev. Biophys. Chem.* 18: 187-221 (1989). For the influenza virus, the hemagglutinin (trimer) HA peptide N-terminal segment (a hydrophobic helical sequence) is exposed due to a conformational change induced by acidic pH in the endosomes (pH 5-6), inserts into the target membrane, and mediates the fusion between the virus and the target endosomal membrane. Weber *et al.*, *J. Biol. Chem.* 269: 18353-58 (1994). Recently, several amphipathic helix-forming oligopeptides have been designed to imitate the behavior of the viral fusion peptide. See, for example, Haensler and Szoka, *Bioconj. Chem.* 4: 372-79 (1993).

Cationic liposome preparations can be made by conventional methodologies. See, for example, Felgner *et*

al., *Proc. Nat'l Acad. Sci USA* 84:7413 (1987); Schreier, *J. of Liposome Res.* 2:145 (1992); Chang *et al.* (1988), *supra*. Commercial preparations, such as Lipofectin® (Life Technologies, Inc., Gaithersburg, Maryland USA), also are available. For some recent reviews on methods employed see Wassef *et al.*, Immunomethods 4: 217 - 222 (1994) and Weiner, A. L., Immunomethods 4: 217 - 222 (1994).

It is possible to control the therapeutic availability of the encapsulated agent by varying liposome size, the number of bilayers, lipid composition, as well as the charge and surface characteristics of the liposomes. The liposomal preparation could contain one or more adjuvants. Furthermore, a carrier protein such as serum albumin can be added.

4. Delivery of the liposome preparation

In general, the dosage of administered liposome preparation will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Dose ranges for particular formulations can be determined by using a suitable animal model.

Liposomes may be administered to a subject intravenously, intraperitoneally, intrathecally, intramuscularly or subcutaneously. See, for example, Kim, *supra*, Bakker-Woudenberg *et al.* (1993), *supra*, Allen *et al.* (1993), *supra*, and Fielding *et al.*, *Clin. Pharmacokinetics 21*: 155 (1991).

For purposes of therapy, antibodies or fragments are administered to a mammal in a therapeutically effective amount. An antibody preparation is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient mammal. In particular, an antibody preparation of the present invention is physiologically significant

if its presence invokes a humoral and/or cellular immune response in the recipient mammal.

5. Cytokines

The vaccines of the present invention comprise cytokines. Examples of cytokines include the interferons (INFs) such as INF-gamma, interleukins (ILs), M-CSF, GM-CSF, and tumor necrosis factor. INF-γ induces macrophages, as well as cell-surface class II histocompatibility antigens on lymphoid cells and monocytes. See, for example, Klegerman *et al.*, "Lymphokines and Monokines," in BIOTECHNOLOGY AND PHARMACY, Pezzuto *et al.* (eds.), pages 53-70 (Chapman & Hall 1993), and Roitt *et al.*, IMMUNOLOGY, 3rd Edition, pages 7.8-7.14 (Mosby 1993). IL-2 is a T cell growth factor and a stimulator of natural killer cells and tumor-reactive T cells. *Id.* Thus, INF-γ and IL-2 are preferred cytokines for the augmentation of the immune response.

6. Examples

ONCOVAX MATERIALS:

Mouse antigen 38cId
DMPC: Survival Tech Lot RD 1426
MSA 25%): Biocell Laboratories, CAlot#4002160
IL-2: Survival Tech. Lot #RD 1534 @ 9.38 mg/ml)
OTx Buffer
PEG

To 50 - 200 mg of DMPC or DMPC/DMPG ata ratio of 4/1, the following is added such that the final volume is between 0.4 - 1.0 ml:

--0.3 - 10 mg of antigen, i.e. 38cId
--0.0 - 7 x 10^6 IU of IL-2
--0.0 - 12 mg mouse serum albumin

Example 1 (freeze-thaw procedure for preparation of the vaccine):

Mix aqueous components. Add to the powdered lipid in a 5 mL vial glass vial. Warm for 10 minutes in a 35-39°C water bath. Vortex for 30 seconds. Bath sonicate at 25-45°C for 15 minutes. Freeze the vials in a dry ice/ethanol bath at -80°C for 15 minutes. Thaw in a 35-39°C water bath for 10 minutes. Repeat the vortexing, sonication, freezing and thawing steps a total of three (3) times. Add aqueous buffer to dilute as necessary. The sample may be washed by centrifugation. Centrifuge at 12,000 rpm for 20 minutes. Remove supernatant and wash two more times.

Example 2 (sonication-fusion procedure for preparation of the vaccine):

Hydrate the lipid in aqueous buffer at a concentration of 100-300 mg/mL. Sonicate in a bath sonicator at 30-45°C until clear. Sterile filter through a 0.2 micron filter. Add antigen, IL-2 and serum albumin. Cool sample 4-15°C. This may be temperature cycled any number of times from -80°C to 15°C as the low temperature to 23°C to 50°C as the high temperature. The sample may be diluted as necessary, and washed by centrifugation as in Example 1.

Example 3 (PEG-fusion procedure):

Hydrate the lipid in aqueous buffer at a concentration of 100-300 mg/mL. Sonicate in a bath sonicator at 30-45°C until clear. Sterile filter through a 0.2 micron filter. Add antigen, IL-2 and mouse serum albumin. Mix with an equal volume of PEG solution of MW 1,000 on up to 20,000. The PEG solution should be between 6% to 60% w/v. Following an incubation at 4°C to 25°C for one to 24 hours, washing by centrifugation will remove the PEG and unincorporated active ingredients.

Example 4 (extrusion procedure):

The sample from Examples 1-3 may be size-reduced by extrusion through a 1.0, 0.4 and 0.2 micron polycarbonate filter. The final size should be between 100 - 200 nm.

The vaccine products under this disclosure are called OncoVAX. Each OncoVAX preparation as well as the (KLH-Id) control was analyzed by determining the amount of antigen (Id), IL-2 and lipid as well as the size of OncoVAX particles.

Final concentrations (ranges) of the structural components of OncoVAX were as follows:

Table 1

Structural Component	Example #1	#2	#3	#4	Control (KLH-Id)
Lipid (mg/mL)	20-60	3-30	20-60	20-60	none
Antigen Id mcg/mL	1-200	1-2,000		1-200	500-1,000
IL-2 (IU/mL) $x10^4$	1-200	1-200		1-200	none
Mean Size (micron)	2-4	1-2		0.1-0.3	Soluble (no particle)

The following are examples of the characterization of Oncovax and the mouse studies exhibiting the antitumor immunity, effective liposomal dose with respect to antigen and IL-2 content, humoral and cellular responses elicited by liposomal vaccines, and the effect on in vivo T cell depletion.

The antigen concentration was determined by a sandwich ELISA method where to unknown antigen bound to rabbit anti mouse IgM a biotinylated rabbit anti mouse IgM was added. To this streptavidin-Europium was added and the Europium fluorescence was measured.

Example 5 (Characterization of a typical Oncovax preparation.)

Samples were rapidly frozen from room temperature between copper planchets without cryoprotectant in liquid propane and replicated in a Balzers freeze-fracture unit, and viewed on a Philips 300 electron microscope. Figure 5A reveals the multilamellar liposomes formed. The mean size is roughly 3.0 microns, as determined by single-particle optical sensing (SPOS). Figure 5B reveals a surface texture with numerous bulges and abrupt changes in the ripple patterns. A smooth ripple pattern is characteristic of DMPC liposomes at room temperature, as seen in Figure 5C, the control DMPC liposomes with mouse serum albumin. Bar=0.4 microns.

Example 6 (Immunity test)

To determine whether liposomal formulation of Id could produce the acquisition of protective antitumor immunity, ten syngenic C3H/HEN mice per group were immunized i.p. with liposomal Id or control Id preparations, or with 50 ug 38C13-derived Id in PBS in a volume of 0.2ml. Two weeks later the mice were challenged with a lethal dose of $2x10^3$ 38C13 cells. Statistical comparisons of survival were made on the basis of nonparametric mantel-log rank p values. Mice surviving greater than 90 days after tumor challenge were euthanized and reported as long term survivors. Immunization with liposomal Id demonstrated significantly prolonged survival as well as protection (30%).

Example 7 (Optimization of liposomal vaccine potency and comparison of potency with KLH conjugated Id vaccine.)

Serial dilutions of input Id antigen were made to prepare for liposomal vaccines which were otherwise identical. The actual amounts of incorporated Id were determined for each vaccine after preparation as outlined previously. Id-KLH was prepared by gluteraldehyde conjugation at a 1:1 ratio of Id and KLH and the dose of

Id administered per animal as indicated in parenthesis. A clear dose dependent effect on the induction of protective antitumor immunity was observed, with mice receiving liposomal vaccine formulations delivering 40, 10, and 2 ug Id per mouse demonstrating significantly superior survival compared with controls immunized with free Id. Mice immunized with liposomal vaccines delivering 0.4 ug Id per mouse were not protected from subsequent tumor challenge.

Example 8

The potency of a representative liposme vaccine containing low amount of Id, compared with serial dilutions of ID-KLH in PBS was examined. Previous studies have determined 50 ug Id in the conjugate formulation to be the optimal dose. Ten mice per group immunized with Id-KLH containing 50, 10, or 2 ug Id per mouse demonstrated 40, 30, and 0 percent protection, respectively, compared with nine mice immunized with a liposomal vaccine containing 2 ug ID, which demonstrated 33% protection from subsequent lethal dose tumor challenge (log rank p=0.007 compared with Id-KLH 2 ug Id dose).

Example 9

The requirement for IL-2 as a component of the liposomal Id vaccine formulation was investigated by preparing several formulations with serial dilutions of input IL-2, holding the other components constant. Mice immunized with the resulting liposomal formulations, all containing a dose of 40 ug Id, were used to immunize mice. Two weeks later all mice were challenged with 2x 10^3 38C13 cells from a single preparation of tumor and followed for survival. The log rank p values refer to comparisons against three Id groups. The survival patterns of these mice following lethal dose tumor challenge shows a clear IL-2 dose-dependence on the induction of protective antitumor immunity. Other

experiments demonstrating the failure of liposomal Id vaccines not containing IL-2 to induce any significant antitumor immunity support the conclusion that IL-2 is a critical component of the vaccine formulation, although liposomal vaccines containing 1/10 the amount of input IL-2 were capable on inducing significant protective antitumor immunity (log rank p 0.004 vs. free Id).

Example 10

In an effort to investigate the cellular mechanism by which liposomal Id vaccines promote the acquisition of protective antitumor immunity, we first determined serum anti-idiotypic antibody levels elicited by the various liposomal vaccine formulations containing different doses of Id in the mice. Individual serum samples were assayed for binding to Id-coated microtitered plates in a direct ELISA. The specificity of the antibody response for idiotype was demonstrated by the lack of binding to control IgM proteins. Serum samples were collected from five individual mice per group two weeks after immunization, just prior to tumor challenge, and the mean anti-idiotypic antibody levels are shown. A clear dose dependent effect of the liposome-entrapped Id was apparent, with mean anti-idiotypic antibody levels of 15, 7, 1, and 0.1 ug/ml detectable by ELISA. This demonstration of humoral response specific for idiotype in the three liposomal vaccine groups containing 2, 10, and 40 ug Id stood out in stark contrast to free Id, which failed to induce any detectable anti-idiotlypic antibody even in a single immunized mouse. However, the mean levels of anti-idiotypic antibody elicited by liposomal Id vaccines was considerably less than that elicited by ID-KLH (55 ug/ml serum).

Example 11

Because the magnitude of the anti-idiotyic antibody response did not correlate entirely with the relative levels of protection induced by liposomal Id vaccines and

the Id-KLH, we also examined evidence for idiotype-specific T cell activation. Splenocytes obtained from 2-3 mice per group which had been immunized i.p. as indicated two weeks earlier were pooled and enriched by T cells passed over nylon wool and placed in 96 well bottomed microliter plates with Id at various concentrations (200 ul, $2x10^5$ cells/well). Irradiated (2000 rads) spleen cells from normal syngenic mice were also added to splenocyte cultures ($2x10^5$) as a source of antigen presenting cells. Cultures were maintained at 37°C, 5% CO_2 for 5-7 days, and 18-24 hours before harvesting 1 uCi [^{3}H-thymidine] (2Ci/mmol, New England Nuclear Research Products, Boston, MA) in 50 ul medium to each well. Incorporated radioactivity was measured in an LKB 1205 beta plate liquid scintillation counter. All determinations were performed in quadruplicate and the data are presented as the mean CPM plus standard error of the mean. Splenic T cells obtained from mice given a single immunization with a liposomal Id vaccine, empty liposomes, free Id, or Id-KLH two weeks earlier were assayed in vitro for proliferative responses to various doses of Id. The representative experiment demonstrates a significant T cell proliferative response to Id but not among groups primed with empty liposomes or free Id. These results are also particularly revealing, because such evidence of T cell activation has never been observed after immunization with Id-KLH and was not observed with an Id-cytokine fusion protein.

Example 12

To definitively establish the role of idiotype-specific T cells in the effector phase of induced protective antitumor immunity, we tested the effect of T cell subset depletion in vivo in immunized mice. Mice were immunized with a single liposomal Id vaccine preparation two weeks after immunization, eight mice per group were randomly assigned to receive treatment with

depleting mAb specific for either CD4+ ((GK1.5, ammonium sulfate purified assightese from the BRMP pre-clinical repository, Frederick, MD), CD8+ T cells (53.6-72, ammonium sulfate purified assightese from the BRMP pre-clinical repository, Frederick, MD), a combination of the two antibodies, or with normal rat IgG (Sigma, St.Louis, MO) every other day for three doses, just prior to challenge with a single preparation of tumor. Three weeks after immunization, all mice were challenged i.p. with 2x 10^3 38c13 cells from a single preparation of tumor and were followed for survival. Depletion of lymphocyte subsets was assessed one and two weeks after final treatment by flow cytometric analysis of spleen cells from normal mice treated with monoclonal antibodies in parallel. For both timepoints of analysis, greater than 95% depletion of the appropriate subset was achieved with normal levels of the other subsets. As shown, depletion of either CD4+ or CD8+T cells among immunized mice was associated with marked reduction of protective antitumor immunity (log rank p=0.012 for either group vs. liposomal Id immunized, normal rat Ig-treated mice). Although mAb treated groups were not significantly different compared with control mice immunized with free Id (log rank p=0.09 and 0.16, respectively, vs. free Id). Combined treatment with anti-CD4 and anti-CD8 mAbs did not result in further abrogation of protection (log rank p=0.10 vs. free Id). Thus, it appears clear that there is an absolute requirement for both CD4+ and CD8+ effector T cells in liposomal Id vaccine induced protective antitumor immunity.

Example 13

As an initial step towards testing liposomal Id immunization against previously established tumors we performed experiments in which tumor challenge was performed first, followed by vaccination later the swne day. For these studies we modified an existing protocol vaccination against subcutaneous 38C13 tumors which

required a non-curative dose of cyclophophamide (CTX) chemotherapy on day ten to retard the growth of this virulent tumor. Mice were injected with 10^4 tumor cells subcutaneously in the flank and then randomly assigned to immunization with liposomal Id, liposomal control Id vaccines, or PBS i.p. later the same day. The subcutaneous route of tumor inoculation was used because of the availability of tumor size monitoring as a surrogate endpoint for survival, and by day ten all mice developed macroscopic, palpable tumor masses of approximately 1 cm diameter. CTX administration (75mg/kg i.p.) was associated with complete disappearance of tumors which was uniformly transient in all control mice but durable in a modest but significant proportion of mice immunized with liposomal Id vaccines (log rank p=0.01 for pooled liposomal Id vs. control groups).

Table 2. Therapeutic effect of Lip Id vaccines against a large s.c. tumor inoculum.

Exp.	Immunogen	No. Survivors/ Total no. mice
1	Lipo Id (10μg)	2/10
	Lipo control Id (10μg)	0/5
	PBS	0/5
2	Lipo Id (20μg)	3/10
	PBS	0/10

C3H mice were injected with 10^4 38C13 tumor cells s.c. and then immunized i.p. as indicated later the same day (day 0). All groups received CTX 75 mg/kg i.p. on day 10. Mice surviving >60 days without tumor relapse were apparently cured.

Although the foregoing refers to particular embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention.

WHAT IS CLAIMED IS:

1. A vaccine comprising a liposome preparation comprising

a) at least one B-cell malignancy-associated antigen;

b) IL-2, alone or in combination with at least one other cytokine; and

c) at least one type of lipid molecule.

2. A vaccine according to claim 1, wherein said antigen comprises all or part of an antibody associated with or produced by a malignant B-cell.

3. A vaccine according to claim 1, wherein said malignant B cell is associated with lymphoma.

4. A vaccine according to claim 1, wherein said malignant B cell is associated with chronic lymphocytic leukemia.

5. A vaccine according to claim 1, wherein said malignant B cell is associated with multiple myeloma.

6. A vaccine according to claim 2, additionally comprising tumor-associated antigen that is not an antibody or antibody fragment.

7. A vaccine according to claim 6, wherein said tumor-associated antigen is MUC-1, EBV antigen or an antigen associated with Burkitt's lymphoma.

8. A vaccine according to claim 1, further comprising a B-cell antigen produced by or associated with non-malignant B-cells.

9. A vaccine according to claim 8, wherein said B-cell antigen is a class 1 or class 2 HLA antigen.

10. A vaccine according to claim 1, wherein said at least one other cytokine is selected from the group consisting of M-CSF, GM-CSF, and IFN-gamma.

11. A vaccine according to claim 1, wherein said lipid molecule is selected from the group consisting of phospholipid, glycolipid, cholesterol, and derivatives of said lipids.

12. A vaccine according to claim 1, further comprising a carrier protein.

13. A vaccine according to claim 12, wherein said carrier protein is albumin.

14. A vaccine according to claim 1, further comprising an adjuvant.

15. A method for inducing humoral and cellular immune responses against malignant B-cells in a mammal, comprising administering to said mammal a vaccine according to any one of claims 1-14.

FIGURE 1A

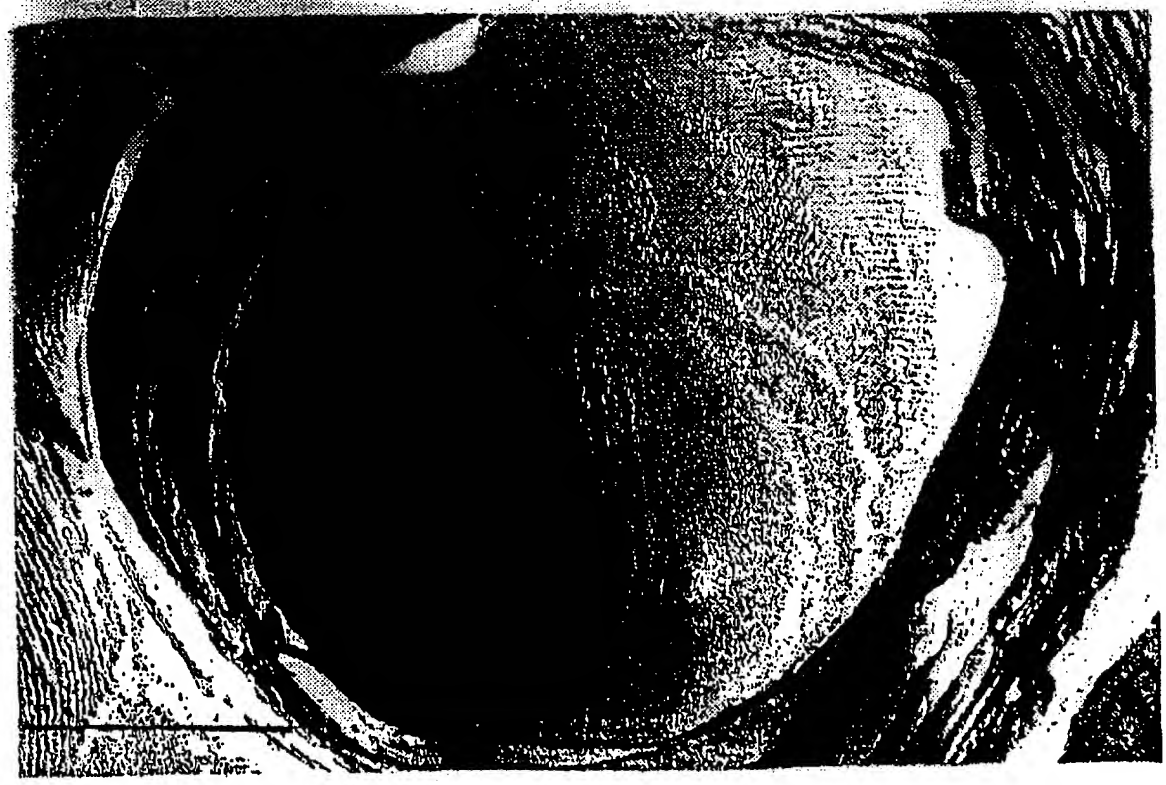
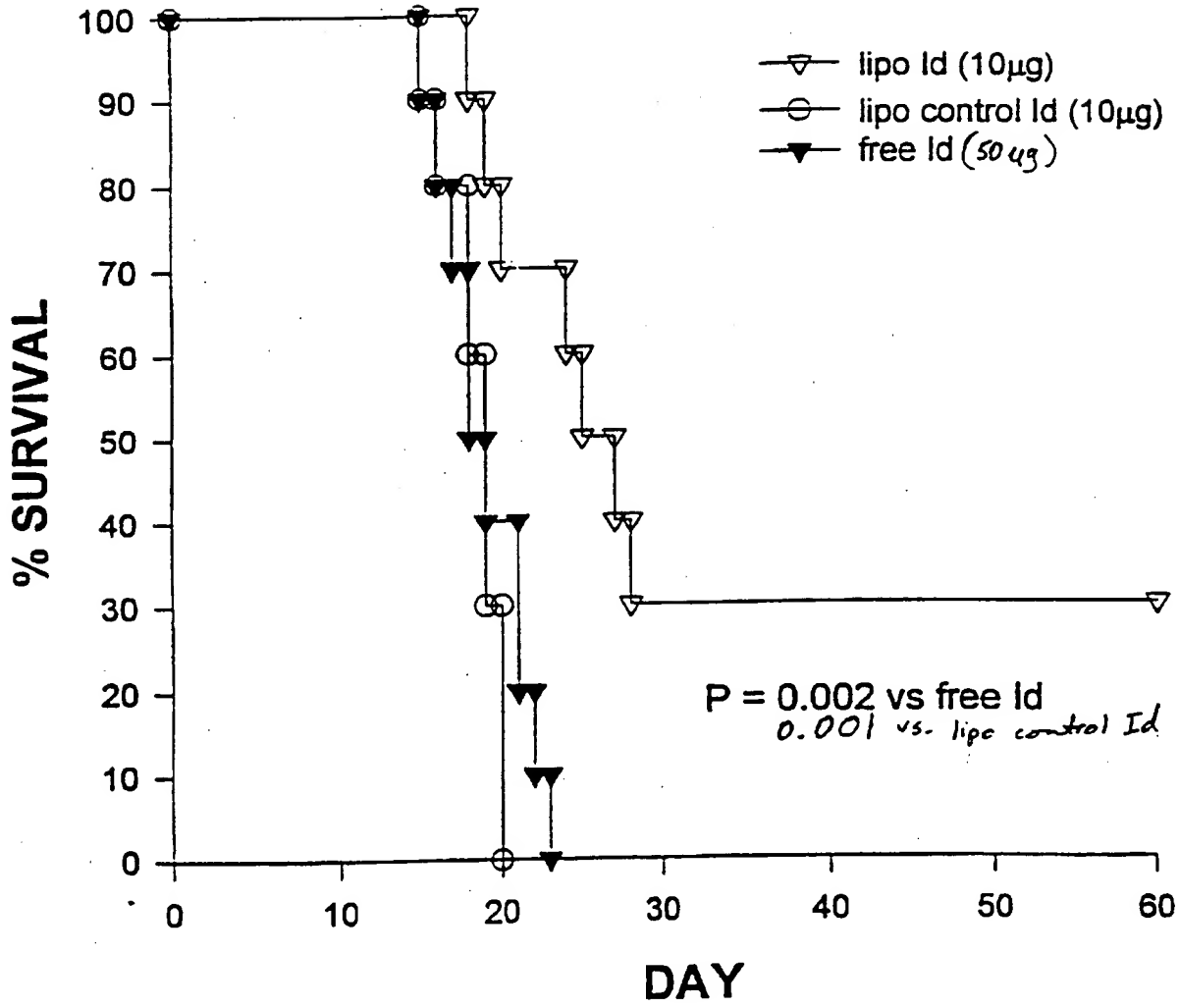


FIGURE 1B

FIGURE 1C

FIGURE 2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90
80
70
60
50
40
30
20
10
0
% SURVIVAL
0
10
20
30
40
50
60
DAY
lipo Id (10µg)
lipo control Id (10µg)
free Id (50 µg)
P = 0.002 vs free Id
0.001 vs. lipo control Id

FIGURE 3

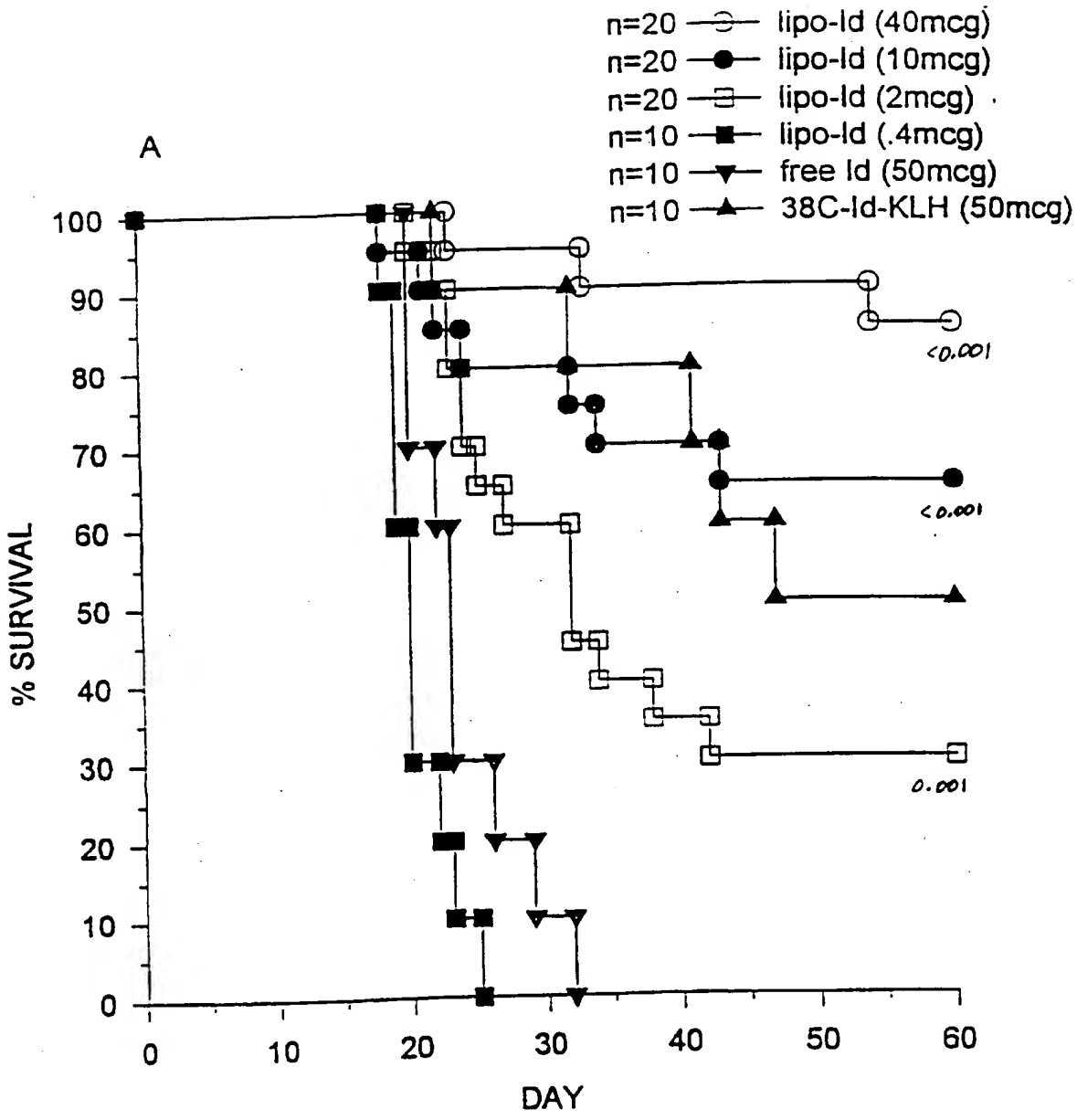
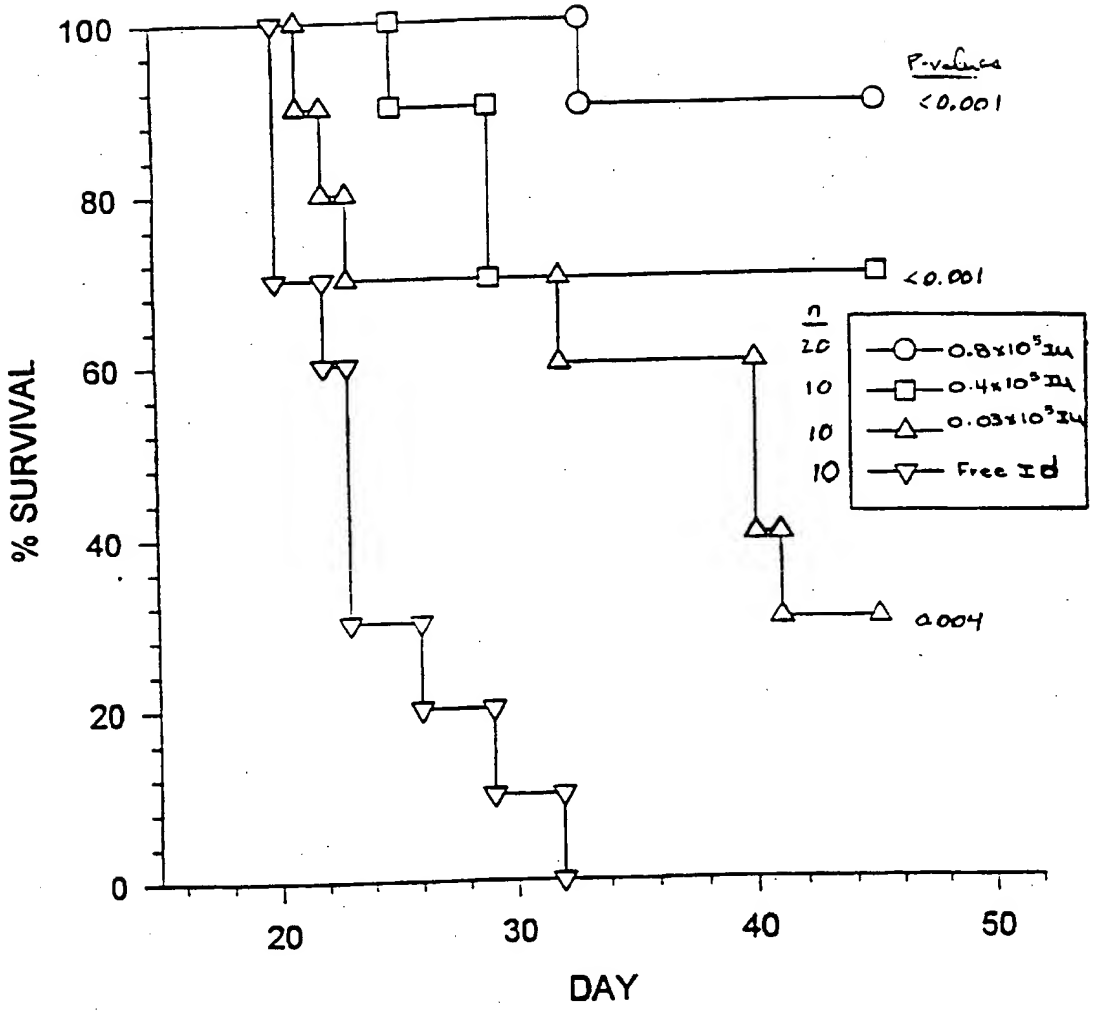

A
n=20 lipo-ld (40mcg)
n=20 lipo-ld (10mcg)
n=20 lipo-ld (2mcg)
n=10 lipo-ld (.4mcg)
n=10 free ld (50mcg)
n=10 38C-ld-KLH (50mcg)
% SURVIVAL
DAY
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
<0.001
<0.001
0.001

FIGURE 4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60
40
20
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% SURVIVAL
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30
40
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10
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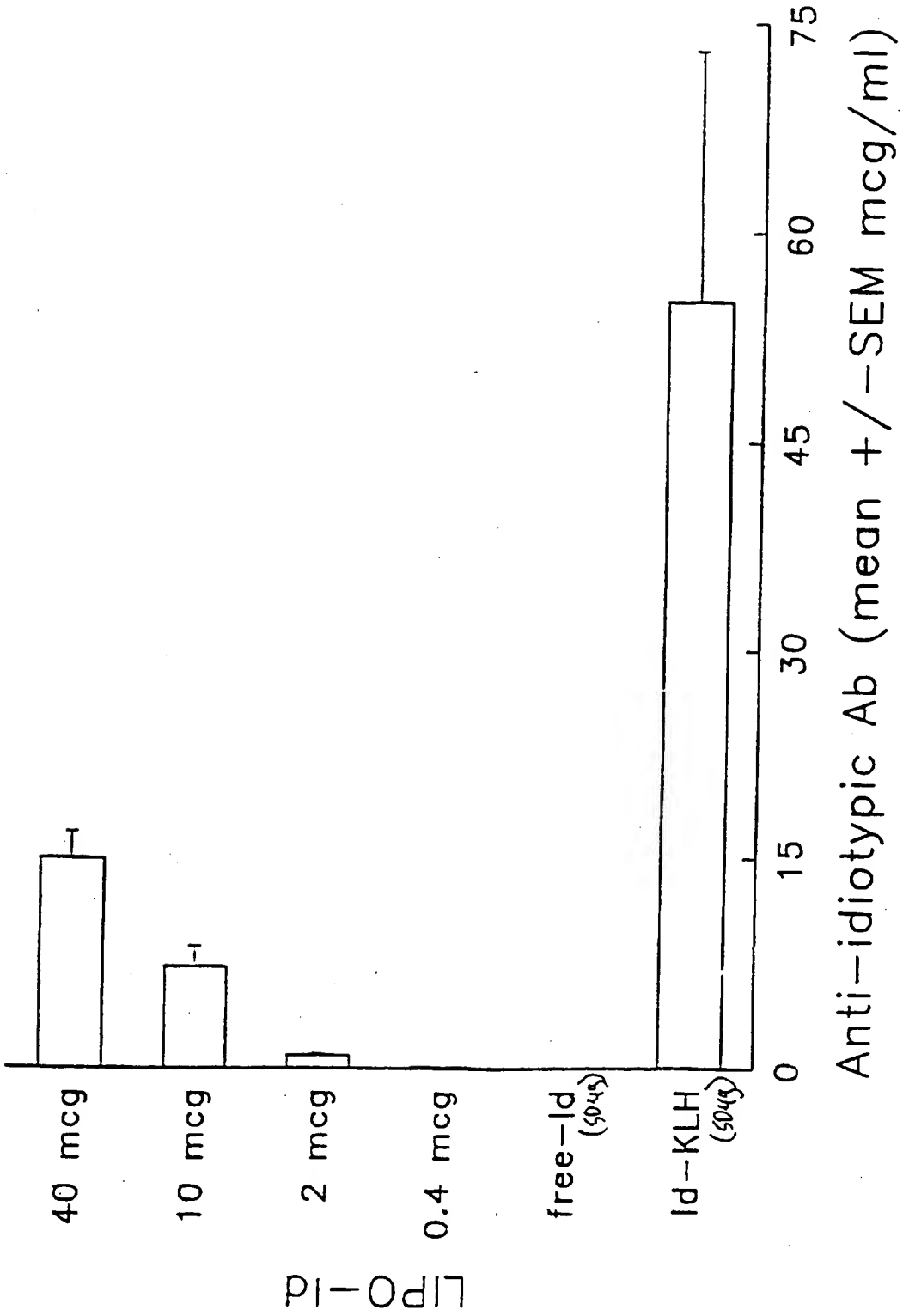
0.4x10^5 IU
0.03x10^5 IU
Free Id

FIGURE 5


LIPO-Id
40 mcg
10 mcg
2 mcg
0.4 mcg
free-Id (50µg)
Id-KLH (50µg)
0
15
30
45
60
75
Anti-idiotypic Ab (mean +/-SEM mcg/ml)

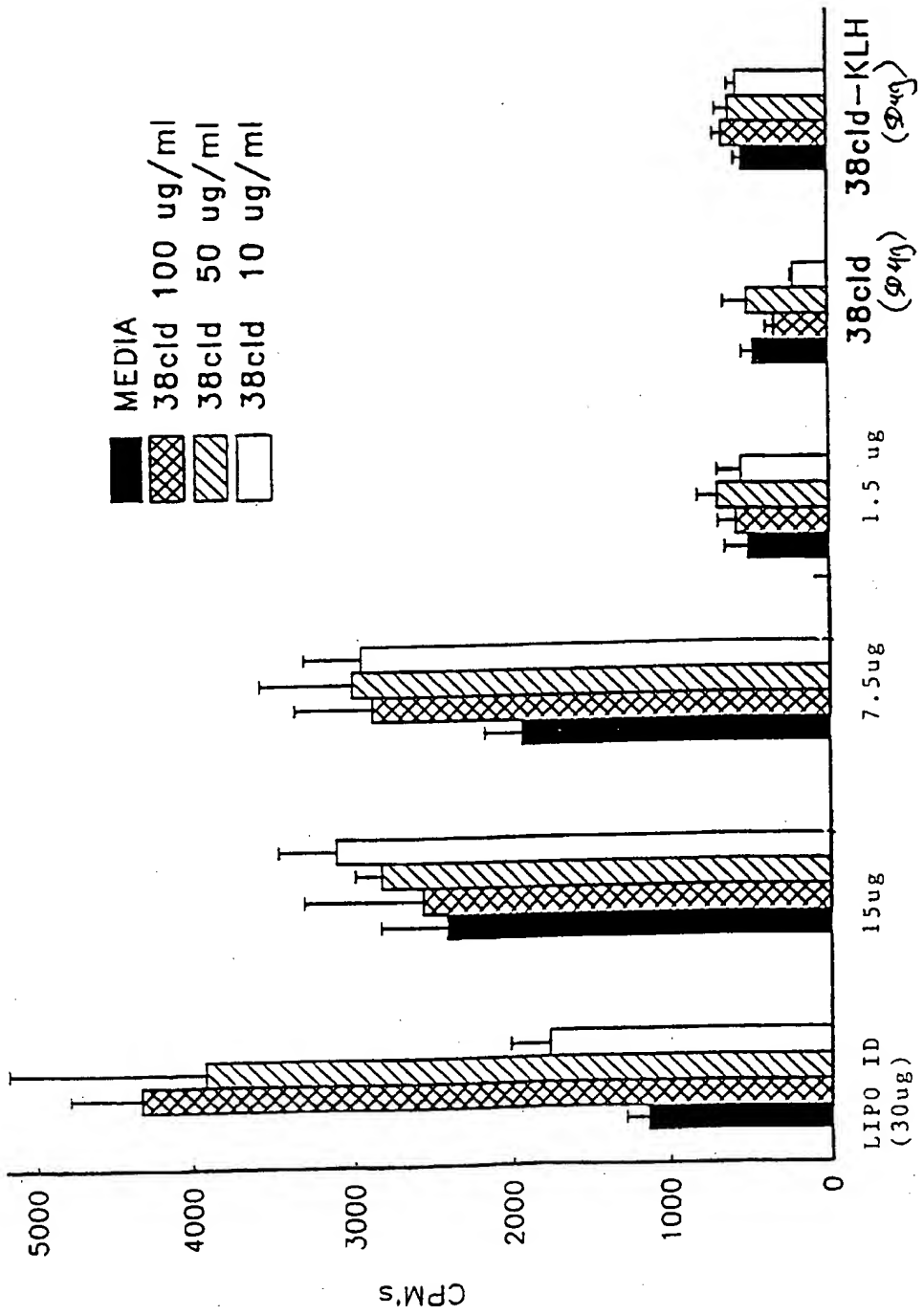
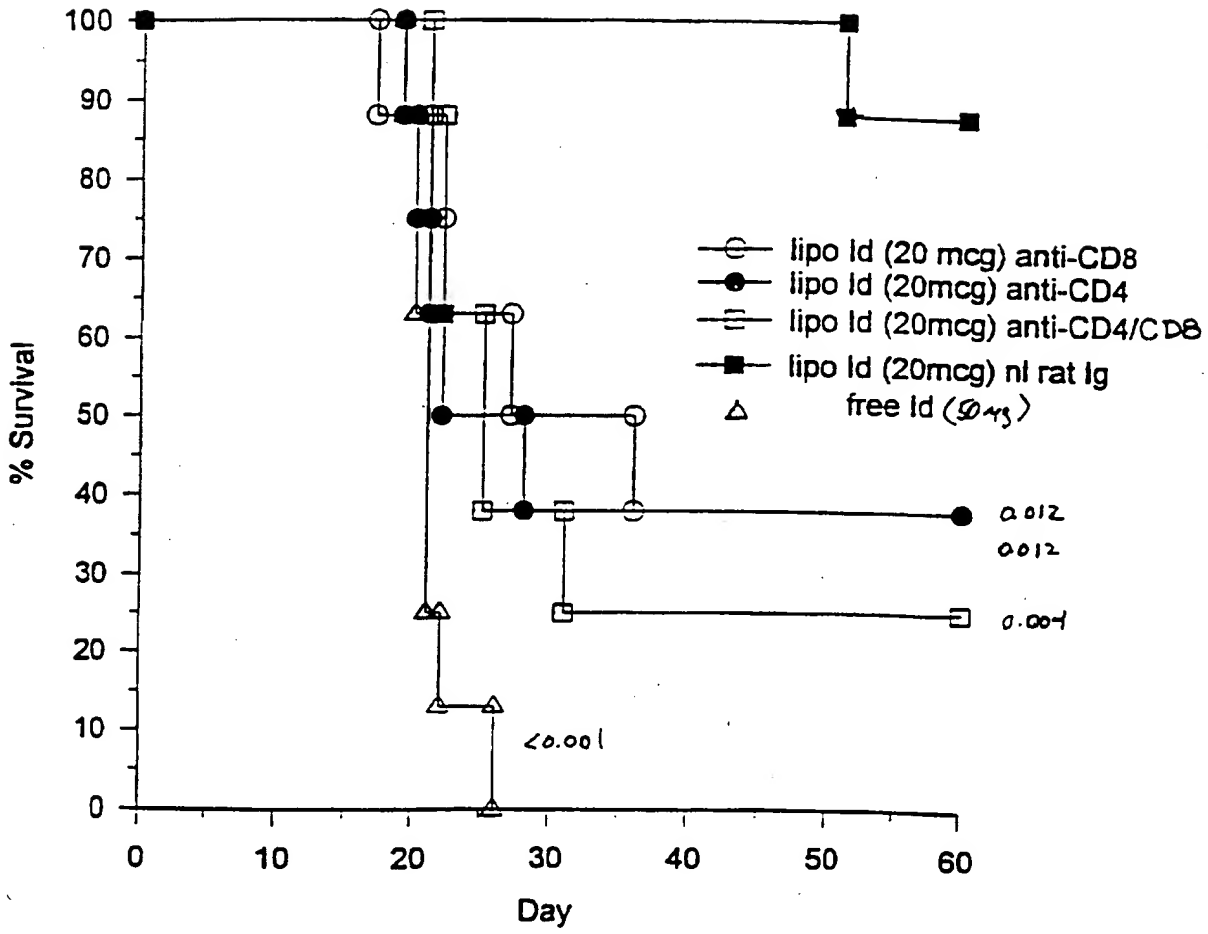

MEDIA
38cld 100 ug/ml
38cld 50 ug/ml
38cld 10 ug/ml
CPM's
5000
4000
3000
2000
1000
0
LIPO ID (30ug)
15ug
7.5ug
1.5 ug
38cld (Φ49)
38cld-KLH (Φ49)

FIGURE 6

FIGURE 7


100
90
80
70
60
50
40
30
20
10
0
% Survival
0
10
20
30
40
50
60
Day
lipo Id (20 mcg) anti-CD8
lipo Id (20mcg) anti-CD4
lipo Id (20mcg) anti-CD4/CD8
lipo Id (20mcg) nl rat Ig
free Id (5045)
0.012
0.012
0.004
<0.001

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02351

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 39/00, 39/39, 39/395, 45/05, 9/127
US CL : 424/130.1, 184.1, 85.1, 277.1, 450
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/130.1, 184.1, 85.1, 277.1, 450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Stedman's Medical Dictionary

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GEORGE et al. Idiotypic Vaccination as a Treatment for a B cell Lymphoma. J. Immunol. 15 September 1988. Vol 141. No. 6. pages 2168-2174, especially abstract and discussion.	1-3, 11-15
Y	LARCHIAN et al. Liposomal Transfection of Murine Bladder Cancer Cells(MBT-2) with Interleukin-2(IL-2). J. Cell. Biochem. 10 March - 04 April 1995. Suppl 21A, page 424, entire abstract.	1-15
Y	EPSTEIN et al. Clinical Consequences of Epstein-Barr Virus Infection and Possible Control by an Anti-Viral Vaccine. Clin. Exp. Immunol. 1983. Vol 53. No. 2. pages 257-271, especially abstract.	2, 6, 7, 14, 15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 APRIL 1997	Date of mailing of the international search report 09 JUN 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GEETHA P. BANSAL Telephone No. (703) 305-3955

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02351

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GREGORIADIS, G. The immunological Adjuvant and Vaccine Carrier Properties of Liposomes. J. Drug Targeting. 1994. Vol 2. No. 5. pages 351-356, entire article.	1-15
Y	ARTHUR et al. Macaques Immunized with HLA-DR are Protected from Challenge with Simian Immunodeficiency Virus. J. Virol. 1995. Vol 69. No. 5. pages 3117-3124, abstract.	1, 8, 9
Y	CHAN et al. Immunization with Class I Human Histocompatibility Leukocyte Antigen can Protect Macaques against Challenge Infection with SIVmac-32H. AIDS. March 1995. Vol 9. No. 3. pages 223-228, abstract.	1, 8, 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02351

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Medline, Biosis, Scisearch, Embase, WPIDS, Cancerlit
search terms: antiidiotypic antibody(ies), vaccine(s), liposome, B cell malignancy, tumor, cancer, cytokine, EBV antigen, muc-1, multiple myeloma, burkitt's lymphoma